REMARKS

Claims 1-20 are pending in the application, and claims 9 and 16-20 have been withdrawn from consideration pursuant to a restriction requirement. Claims 1-3 and 5 were rejected under 35 U.S.C. § 102(b) and claims 4-8 and 10-15 were rejected under 35 U.S.C. § 103(a). Each of the rejections is addressed below.

Information Disclosure Statements

Applicants thank the Examiner for considering the Information Disclosure Statements filed on December 19, 2007 and January 22, 2008. With regard to the disclosed references, the Examiner stated in the June 26, 2009 Office action "the submissions are in compliance with the provisions of 37 CFR 1.97." However, the Examiner did not initial three of the references on the December 19, 2007 Form PTO-1449. Therefore, it is unclear whether the following three references have been considered: CZ 223295, DE 101 18 852, and Arya et al. Submitted herewith is a new Form PTO-1449 including only these three references. Applicants respectfully request that the Examiner consider these references and return the Form PTO-1449 initialed.

Amendments to the claims

Claim 1 has been amended to specify that the nanoparticles are of an average size of 500 nm or less. Claim 7 has been amended to specify nanoparticles of 50 to 300 nm. Support for these amendments can be found, e.g., at paragraph 138 of the application as published and in the Examples.

Rejections under 35 U.S.C. § 102(b)

The Examiner rejected claims 1-3 and 5 as being anticipated by Laus et al. (J. Control. Rel. 2001, 72, 225) as evidenced by the specification and test methods for EUDRAGIT L100-55, Degussa. However, the particles described by Laus do not fall within the range of sizes specified by amended claim 1.

Laus describes microspheres prepared by dispersion polymerization (see title and introduction). Although Laus states that, in general, dispersion polymerization is a process that

generates latex microspheres in the 0.5 to 20 micron diameter range, there is no disclosure or suggestion in Laus that dispersion polymerization using the conditions, materials, and methods specifically described by Laus achieves or is capable of achieving the lower limit of 0.5 micron diameter.

Dispersion polymerisation, as described in Laus, is a process that features the polymerization of a monomer dissolved in an organic diluent in the presence of a polymeric stabilizer. This polymerization process generally generates latex microspheres in the 0.5-20 micron diameter range, depending on the monomer, organic diluent, and polymeric stabilizer employed. Laus describes the synthesis of two distinct series of microspheres, made of polystyrene (PS) and polymethylmethacrylate (PMMA), in which the steric stabilizers are respectively hemisuccinated polyvinylalcohol and Eudragit L100/55 (a methacrylic acid/ethylacrylate 1/1 statistical copolymer); see page 282, paragraph 2. The obtained microspheres have a particle size of between 0.99 and 6.73 micrometres, i.e., 990 nm to 6,730 nm. An extensive study of these specific microspheres was performed in a subsequent paper which, similar to Laus, includes inventors named on the present application as authors (Sparnacci et al., J. Biomater. Sci. Polymer Edn. 2005, 16, 1557; a copy of which is submitted herewith). Particle sizes ranging from 0.65 to 10.18 micrometers were obtained (see Table 2 of Sparnacci). Applicants find that particles with diameter lower than 0.65 micron (650 nm) were not obtained by dispersion polymerisation of methyl methacrylate employing Eudragit L100/55 as polymeric stabilizer, i.e., the method described in Laus.

The microspheres of Laus are produced using a different process from the process by which the nanospheres of the instant application are produced. As explained above, Laus uses a dispersion polymerization process that features the polymerization of a monomer dissolved in an organic diluent in the presence of a polymeric stabilizer. Laus does not enable the production of particles less than 650 nm in diameter. The instant application describes the synthesis of polymeric nanoparticles by emulsion polymerization with diameters 0.50 microns or lower. This process features the polymerization of an organic monomer dispersed (not dissolved) in an aqueous (not organic) medium. The obtained particles are 500 nm or less in diameter and can be stabilized employing a surfactant, a polymeric stabilizer, a water-soluble co-monomer, or an

ionic free radical initiator.

Laus describes a different process that results in particles larger than the nanoparticles defined in the instant claims. Particles as small as 500 nm or less are not enabled by the process described by Laus. Therefore, the rejection for anticipation may be withdrawn.

Rejections under 35 U.S.C. § 103(a)

Claims 1 and 7 are rejected as being obvious over Laus. The Examiner based the rejection on the conclusion that Laus teaches a similar composition as the instant claims with an overlapping particle size range. The claims have been amended to eliminate any overlap between the particles size range of the instant application and the particles disclosed as produced by Laus.

The Examiner interprets Laus as teaching a particle size distribution of 0.5 to 20 microns in diameter. However, this statement in the introduction to Laus is a general statement that latex microspheres produced by dispersion polymerization may be in the 0.5 to 20 micron diameter range. There is no disclosure or suggestion in Laus that the specific microspheres described in Laus can or should be produced in this size range. The experimental results of Laus only support a disclosure of microspheres having a size range of 0.99 to 6.73 micrometers, i.e., 990 to 6,730 nm. The nanoparticles of the instant claims are now defined as having a diameter of 500 nm or less.

The Examiner also concludes that those skilled in the art would have been motivated to select a particle size range that is suitable for administration to animals or humans, and that those skilled in the art would have had a reasonable expectation of success at optimizing the particle sizes on the alleged ground that this would be routine optimization within the purview of those skilled in the art.

However, there is no disclosure or suggestion in Laus that particle sizes of 500 nm or less may be sought or could or should be achieved. On the contrary, Laus suggests that the process of dispersion polymerization produces successful microspheres of an appropriate size range. This size range is above the range now claimed. There is no motivation for the skilled person to work outside the size range described and achieved in practice by Laus.

Even if the skilled person tried to follow the teachings of Laus but ignored the size range

taught by Laus and tried to produce smaller microspheres, it would not be possible to produce spheres as small as 500 nm or less. To produce such small particles it is necessary to ignore the process described by Laus and to use a completely different process as described in the instant application.

As discussed in paragraph 8 of the published application, the aims of the present invention include developing biocompatible polymeric carriers able to reversibly bind and deliver pharmacologically active substances, such as nucleic acids, intact into cells. The invention also aims to develop stealth carriers, able to avoid recognition by the phagocytic cells and to last longer in the blood stream.

The inventors have surprisingly found that emulsion polymerization of the monomers identified in the claims enables the production of core-shell particles that are smaller in size than those contemplated in Laus. The nanoparticles can be functionalized with positive or negative charged groups. This allows them to be tailored for use with a broad range of nucleic acids, proteins with different isoelectric points, and drugs.

Nanoparticles having a size of 500 nm or less are neither anticipated, nor made obvious by Laus. The rejection of claims 1 and 7 for obviousness may, therefore, be withdrawn.

Claim 4 was rejected as being obvious over Laus in view of Schacht et al. (U.S. Patent No. 6,312,727). Claim 4 incorporates the features of claim 1. As discussed above, claim 1 is non-obvious in view of Laus. Similarly, Schacht does not teach nanoparticles with an average diameter of 500 nm or less. Based on this deficiency alone, this rejection may be withdrawn. Moreover, the skilled artisan would not have been motivated to combine the teachings of Schacht and Laus, as the particles taught by each reference are based on incompatible technologies.

Schacht relates to the delivery of nucleic acid material to target cells in biological systems. The product of Schacht is a nucleic acid-containing core portion comprising a cationic polymer material and nucleic acid material, especially DNA contained in an expression vector. This DNA-containing core is then provided with a coating and steric shield containing molecules of hydrophilic polymer material. Thus, Schacht uses the coating to protect the core (containing active ingredient) from the adverse influence of outside materials. The shell provides a

hydrophilic steric barrier to shield the DNA core from interactions with cells and molecules encountered, e.g., while circulating in the plasma after IV administration (Schacht, column 2, lines 26 to 56).

In Laus the outer shell of the particles is described as "soft" (Laus page 280, first paragraph). The active ingredient, e.g. protein, is adsorbed onto the outer surface of the "soft outer shell."

Laus thus uses the shell for a completely different purpose than Schacht. In Schacht the active ingredient is in the core and the shell is used as a protective coating. In Laus the active ingredient is attached to the shell, which is used as a means of anchoring the active ingredient to the core. In view of the different roles played by the core and shell in each of Laus and Schacht the skilled person would not have been motivated to combine these documents. Therefore, claim 4 is non-obvious over Laus in view of Schacht.

Claim 6 was rejected over Laus in view of Jon et al. (Langmuir 2003, 19, 9989-9993). The Examiner asserts that it would have been obvious to incorporate polyethylene glycol methyl ethyl methacrylate as a "coating" since it is alleged to be taught as a suitable polymer for creating non-fouling surfaces by Jon.

However, the context in which Jon discloses the use of polymeric self-assembled mono layers is very different from the context of Laus and from the present invention. As explained in the introduction of Jon, the authors of this article are concerned with the construction of protein- or cell- resistant surfaces for medical or analytical devices. Such devices include biosensors, chip-based diagnostic assays, affinity chromatography columns and biomaterials used for implants and tissue engineering. Jon is thus concerned with devices and hardware. The Examples refer to polymer-coated glass substrates and compare them to unmodified glass substrates. The skilled person would not have been motivated to combine this teaching of Jon concerning medical devices with the teaching of Laus, which relates to microspheres as a protein delivery system. Furthermore, Jon does not teach nanoparticles with a average diameter of less than 500 nm. Therefore, Jon in combination of Laus does not render amended claim 1 obvious. As claim 6 depends from claim 1, the rejection of claim 6 for obviousness can be withdrawn.

Claim 8 was rejected over Laus in view of Melker et al. (U.S. Patent No. 6,974,706). Claim 8 is dependent on claim 1 and, as explained above, claim 1 is non-obvious over Laus. The teachings of Melker are directed to a method for detecting biological conditions through non-invasive analysis of bodily fluid samples, including exhaled breath and blood. Melker is quite clear that it is an essential feature of their method that it is non-invasive. In contrast, Laus describes use of their microspheres as "protein delivery systems." There is no motivation for the skilled person to try to apply the teachings of Melker concerning a non-invasive method to the microspheres of Laus.

Even if the skilled artisan were to consider Melker as relevant to and combinable with the teachings of Laus, the resulting combination does not lead in an obvious manner to the subject matter of the present application. Similar to Laus, the teachings of Melker do not teach nanoparticles of less than 500 nm in average diameter. Therefore, claim 8 is non-obvious over Laus in view of Melker.

Claims 10-15 were rejected over Laus in view of Le Buanec et al. (Biomed. Pharmacol. 2001, 55, 316). The Examiner acknowledges that Laus does not teach the absorption of a pharmaceutically active agent such as an antigen. The Examiner relies on Le Buanec to teach the use of microspheres for the administration of Tat toxoid. However, the particles of Le Buanec are physically different from those of claim 10.

Le Buanec describes the use of chitosan or polylactide-co-glycolide (PLG) formulations in which the Tat inactivated antigen is encapsulated. Page 317 of Le Buanec describes how the particles were prepared. Nanoparticles of chitosan loaded with Tat toxoid (TTx) were prepared according to the procedure described by Roy et al. (Nat. Med. 1999, 5, 387). Polylactide-co-glycolide microparticles enriched with TTx were prepared according to the method described by Benoit et al. (Infect. Immun. 1999, 67, 2643). In the cited papers the preparation process results in the antigen being encapsulated.

Claim 10 defines the pharmacologically active agent as being adsorbed at the surface of the nanoparticles. There would have been no motivation for the skilled person to have combined the teachings of Le Buanec with those of Laus. Also, the teaching of Le Buanec does not

overcome the failure of Laus to disclose or suggest nanoparticles with an average diameter of less than 500 nm, as specified in amended claim 1. Even if the skilled person were to try to combine the teachings of Laus and Le Buanec, Le Buanec describe particles in which an antigen is encapsulated in a particle and not adsorbed on the surface. Accordingly, claims 10-15 are non-obvious over Laus in view of Le Buanec, and this rejection may be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested. Submitted herewith is a Petition to extend the period of reply for two months, to and including November 26, 2009.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: November 25, 2009

Susan M. Michaud

Susan M. Michaud, Ph.D.
Reg. No. 42,885

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

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Core–shell microspheres by dispersion polymerization as promising delivery systems for proteins

KATIA SPARNACCI [1], MICHELE LAUS [1,*], LUISA TONDELLI [2,*], CINZIA BERNARDI [2], LAURA MAGNANI [2], FRANCO CORTICELLI [3], MARCO MARCHISIO [4], BARBARA ENSOLI [5], ARIANNA CASTALDELLO [6] and ANTONELLA CAPUTO [6]

[1] *Department of Environmental and Life Sciences (INSTM), University of Piemonte Orientale, Spalto Marengo 33, 15100 Alessandria, Italy*
[2] *ISOF-CNR, Via Gobetti 101, 40129 Bologna, Italy*
[3] *IMM-CNR, Via Gobetti 101, 40129 Bologna, Italy*
[4] *Department of Morphology and Embriology, University of Ferrara, Via Fossato di Mortara 66, 44100 Ferrara, Italy*
[5] *Istituto Superiore Sanità, Viale Regina Elena 299, 00161 Roma, Italy*
[6] *Department of Histology, Microbiology and Medical Biotechnology, University of Padova, Via A. Gabelli 63, 35122 Padova, Italy*

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Abstract—Functional poly(methyl methacrylate) core–shell microspheres were prepared by dispersion polymerization. An appropriate selection of experimental parameters and in particular of the initiator and stabilizer amount and of the medium solvency power allowed a monodisperse sample as large as 600 nm to be prepared. To this purpose, low initiator concentration, high steric stabilizer amount and a low solvency power medium were employed. The microspheres present a core–shell structure in which the outer shell is constituted by the steric stabilizer which affords carboxylic groups able to interact with basic proteins, such as trypsin, whose adsorption is essentially driven by the carboxylic group density in the microsphere shell. Finally, fluorescent microspheres were prepared for biodistribution studies and shown to be readily taken up by the cells both *in vitro* and *in vivo*. These results suggest that these microspheres are promising delivery systems for the development of novel protein-based vaccines.

Key words: Core–shell microspheres; dispersion polymerization; protein; vaccine; delivery.

*To whom correspondence should be addressed. E-mail: laus@mfn.unipmn.it and tondelli@isof.cnr.it

INTRODUCTION

Due to recent advances in the area of biotechnology, biochemistry and molecular biology, numerous bioactive peptides and proteins are available in large quantities for therapeutic and vaccine purposes. However, most of them have short half-lives *in vivo* because they are easily degraded or denatured in biological fluids and sometimes not efficiently absorbed by the gastrointestinal tract due to their relatively high molecular mass. This implies that multiple administrations are required for their efficacy: for these reasons, there has been a growing need for suitable delivery systems capable of stabilizing and increasing the shelf-life of these molecules and of efficiently delivering them to the desired location [1, 2].

The use of microparticles as drug/vaccine carriers to increase the bioavailability of peptide and protein drugs and vaccines is an expanding research field. The most promising systems described up to now are based on encapsulation or entrapment of proteins into biocompatible polymeric devices [3, 4]. In particular, biodegradable polymeric nano- and microparticles are widely investigated to produce controlled and sustained release of bioactive molecules (for a review see Ref. [5]). Most of the work reported in literature is focused on protein encapsulation inside poly(lactide-*co*-glycolide) (PLG) matrices because of their good biocompatibility, non-toxicity and favourable biodegradation pattern [6, 7]. These polymeric particles have also shown great potential as vaccine adjuvants due to their ability in optimizing antigen presentation and, hence, humoral and cellular response [8–10]. However, some concerns have arisen about the effects of both the encapsulation procedures and PLG degradation on protein stability [11–14].

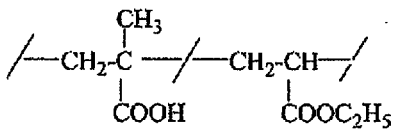
As an alternative to the encapsulation method, there is a completely different conceptual approach in the development of delivery systems, based on core–shell microspheres or nanospheres in which the shell allows docking of the bioactive molecule which can later be released in a biologically active form. Modified PLG microparticles carrying antigens on their surface are able to increase the potency of DNA and protein vaccines in several animal species [15–17]. However, the use of chlorinated solvents and/or surfactants during particle preparation may interfere with the reproducibility of particle size and size distribution, thus affecting their biocompatibility. To overcome these problems, we designed and developed a new type of poly(methyl methacrylate) (PMMA) core–shell microspheres, which were prepared by dispersion polymerization [18–21] using, as the steric stabilizer, a commercial statistical co-polymer of methacrylic acid and ethyl acrylate (Eudragit L 100-55). According to the polymerization mechanism, they present a core mainly constituted of biocompatible PMMA and a shell of Eudragit L 100-55 which, irrespective of its width, strongly drives the microsphere behavior in terms of protein adsorption propensity. Actually, these microspheres were specifically designed for binding biologically active macromolecules on their shell without the need for added surfactants. We also tested the feasibility of this approach by studying the adsorption behaviour of bovine serum albumin (BSA), as a model protein [22]. PMMA is already known to be a well-tolerated polymeric material,

used in bone repair and surgery. In addition, it has been shown to be slowly degradable in the form of nanoparticles and to be an efficient vaccine adjuvant without any observable *in vivo* side effects or toxic reactions [23–25]. On the other hand, Eudragit® co-polymers, already approved for human use, are also highly efficient as steric stabilizers in dispersion polymerization systems to prepare core–shell microspheres bearing functional groups covalently bound to the surface [26].

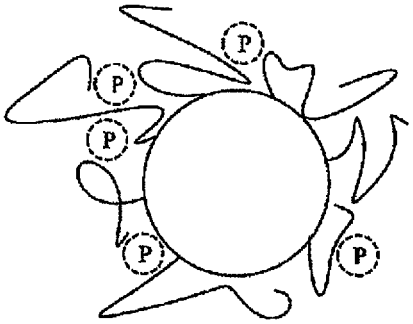
Recent studies indicate that the human immunodeficiency virus type 1 (HIV-1) Tat protein represents a promising candidate for a prophylactic and/or therapeutic vaccine against HIV-1/AIDS [27, 28] and phase-I preventive and therapeutic clinical trials with the Tat protein vaccine have just been completed in Italy. Due to its high cysteine content, Tat is a very labile protein, which oxidizes and, consequently, very rapidly looses its biological activity when handled at room temperature and in light [29]. Since Tat contains a positively charged domain, we have previously studied the adsorption–release behaviour and stability of HIV-1 Tat protein on some of the above core–shell microspheres with size comprised between 1 and 5 μm. We demonstrated that Tat adsorption onto the microsphere surface prevented its oxidation and loss of biological activity, after exposure to light and room temperature, thus greatly simplifying the handling and increasing the stability of this protein vaccine [30]. Irrespective to the microsphere size, the Tat–microsphere complexes were not toxic, and elicited broad antigen-specific immune responses in mice after intramuscular immunization (Ref. [30] and unpublished results). In addition, the immune responses were induced with a low amount of antigen and few booster shots, indicating that these macromolecules are promising delivery systems for the development of protein-based vaccines with increased stability, especially when the native conformation of the molecule is required.

Based on these results, a systematic investigation of the effect of several experimental parameters on the physicochemical characteristics of these innovative core–shell microspheres is reported in the present work, including size, shell structure and protein adsorption. The steric stabilizer, Eudragit L 100-55, whose structure is shown in Scheme 1, is water soluble, depending on the pH of the medium. Accordingly, the formation of protein–microsphere complexes in which the hydrophilic arms, constituting the microsphere shell and presenting carboxylic groups, are able to form a complex with proteins, is shown in Scheme 2.

To elucidate this point, trypsin was chosen as a model protein because its size and isoelectric point are similar to those of the HIV-1 Tat protein. Moreover, to obtain information on microsphere uptake in cellular systems and *in vivo*, a sample



Scheme 1.



Scheme 2.

of yellow/green fluorescent core–shell microspheres was prepared and employed as a fluorescent probe *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

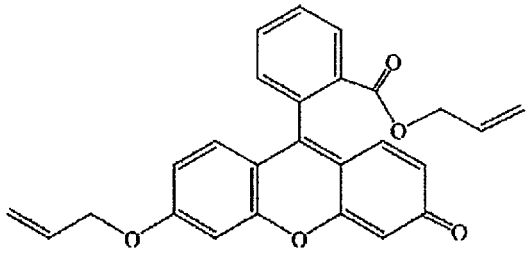
Methanol (99.9%, Carlo Erba) and 2,2′-azobis (isobutyronitrile) (AIBN) (98.0%, Fluka) were used without further purification. Methyl methacrylate (MMA) (99%, Aldrich) was distilled under vacuum immediately before use. The poly(methacrylic acid, ethyl acrylate) 1 : 1 statistical co-polymer (trade name Eudragit L 100-55), kindly supplied by Röhm Pharma as a powder sample, is characterized by a number average molar mass (M_n) of 250 kg/mol. Trypsin (pI = 9.74) was purchased from Sigma.

Synthesis of fluorescent monomer 1

2.0 g fluoresceine (6.0 mmol), 2.0 g calcium carbonate and hydroquinone (trace) were dissolved in 100 DMF and the solution was heated at 60°C. Allyl chloride was added dropwise and the reaction was allowed to proceed for 30 h in the dark. After vacuum evaporation of the solvent, the product was purified by flash column chromatography (silica gel; diethyl ether/ethyl acetate 80 : 20 as eluent). Max λ_{em} = 528 nm (λ_{exc} = 488 nm), yield 53%, (m.p. 123–125°C); MS, m/z (%): 412 (M^+, 100), 371 (10), 287 (20), 259 (15), 202 (7); ^{1}H-NMR (CD_3OD): δ 4.44 (dd, J = 5.9 and 1 Hz, 2 H, O—CH_2—CH=), 4.75 (dd, J = 5.9 and 1 Hz, 2 H, O—CH_2—CH=), 5.08 (m, 2H, CH_2=CH), 5.40 (m, 2H, CH_2=CH), 5.58 (m, 1H, CH_2=CH), 6.10 (m, 1H, CH_2=CH), 6.60 (m, 2H, Ar), 6.98 (m, 3H, Ar), 7.25 (d, J = 1 Hz, 1H, Ar), 7.45 (dd, J = 7.5 and 1 Hz, 1H, Ar), 7.85 (m, 2H, Ar), 8.30 (dd, J = 7.5 and 1Hz, 1H, Ar).

Dispersion polymerization procedure

Four series of samples, **1a–e**, **2a–e**, **3a–c** and **4a–c**, were prepared according to the experimental conditions reported in Table 1.



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Scheme 3.

Table 1.
Experimental conditions for the preparation of sample series **1–5**

Sample	MeOH (wt%)	H_2O (wt%)	MMA (wt%)	AIBN (wt%)	Eudragit L100-55 (wt%)	Yield (%)
1a	87.8	0	10.0	0.2	2.0	64.2
1b	86.0	0	10.0	0.2	3.8	76.2
1c	83.9	0	10.0	0.2	5.9	69.5
1d	82.0	0	10.0	0.2	7.8	70.1
1e	80.0	0	10.0	0.2	9.8	65.3
2a	66.7	21.1	10.0	0.2	2.0	67.5
2b	65.4	20.6	10.0	0.2	3.8	64.6
2c	63.8	20.1	10.0	0.2	5.9	73.0
2d	62.3	19.7	10.0	0.2	7.8	78.6
2e	59.9	19.1	10.0	0.2	9.8	73.1
3a	63.9	20.2	10.0	0.05	5.9	56.6
3b	63.8	20.2	10.0	0.1	5.9	60.1
3c	63.7	20.1	10.0	0.3	5.9	80.5
4a	46.8	39.4	10.0	0.05	3.8	74.8
4b	46.8	39.3	10.0	0.1	3.8	86.7
4c	46.7	39.3	10.0	0.2	3.8	88.1
5a[a]	63.8	20.1	10.0	0.2	5.9	66.3

Based on total recipe (184.0 g). The dispersion polymerization reactions were performed at 60°C for 24 h under continuous stirring, nitrogen atmosphere and a reflux condenser. For samples **2a–e**, **3a–c** and **5a**, the ratio between methanol and water in the solvent mixture is 76 : 24 wt%, whereas for samples **4a–c** it is 54 : 46 wt%.

[a] Reaction performed in presence of 5.0 mg fluoresceine derivative **1**.

As a typical example, the polymerization of sample **1b** is described. 7.36 g Eudragit L 100-55 was dissolved in 200 ml methanol and heated to 60°C with mechanical stirring (stirring speed 300 rpm) under nitrogen atmosphere and reflux condenser. After 30 min, 370 mg (2.25 mmol) AIBN, dissolved in 18.3 g (183 mmol) MMA, was added to the solution and the reaction was allowed to proceed for 24 h. At the end of the reaction, the latex was cooled, filtered through a filter paper and then purified. The following washing procedure was then employed.

(1) The latex was centrifuged at 3000 rpm for 15 min and the supernatant was discharged. (2) The pellet was redispersed in fresh methanol and vigorously mixed for 15 min. (3) Steps 1 and 2 were repeated 4 times to eliminate any residual monomer, initiator and unbound Eudragit. At the end, the sample was redispersed in pure methanol. (4) The obtained latex was centrifuged at 3000 rpm for 15 min and the supernatant was discharged. (5) The pellet was redispersed in HPLC-grade water and vigorously mixed for 15 min. (6) Steps 4 and 5 were repeated 4 times to eliminate any residual methanol. The reaction yield was 76.2%, as determined gravimetrically.

Fluorescent sample **5a** was obtained by reacting fluorescent monomer **1** together with MMA in the dispersion polymerization reaction. In this preparation, 11.0 g Eudragit L 100-55 was dissolved in 200 ml methanol/water (76:24, w/w) and heated at 60°C under mechanical stirring (stirring speed 300 rpm) and nitrogen atmosphere and using a reflux condenser. After 30 min, 370 mg (2.25 mmol) AIBN and 5.0 mg (12 μmol) fluorescent monomer **1**, dissolved in 18.3 g (183 mmol) MMA, were added to the solution and the reaction was allowed to proceed for 24 h. At the end of the reaction, the latex was purified as described above.

Physico-chemical characterization

Microsphere size and size distribution were measured using a Jeol JEM 100CX scanning electron microscope (SEM) operating at an accelerating voltage of 20–30 kV. The samples were sputter-coated under vacuum with a thin layer (10–30 Å) of gold. The magnification was given by the scale on each micrograph. The SEM photographs were digitalized and elaborated by the Scion Image processing program. 150–200 individual microsphere diameters were measured for each sample. Table 2 reports the number-average diameter $\overline{D}_n$, the weight-average diameter $\overline{D}_w$ and the uniformity ratio U [31].

The amount of Eudragit L 100-55 linked to the microsphere surface was determined by back titration of the excess NaOH after complete reaction of the acid groups and microsphere removal by centrifugation. To this purpose, 0.6 g of each microsphere sample was dispersed in 10 ml NaOH (20 mM) at room temperature for 24 h, under nitrogen atmosphere. Then, the microsphere sample was collected by centrifugation and washed twice with 25 ml distilled water. The supernatants were combined and the excess NaOH was titrated with 20 mM HCl under nitrogen atmosphere. ζ-potential measurements were carried out at 25°C on a Malvern Zetasizer 3000 HS instrument, after dilution of microspheres in the presence of 10 mM NaCl solution and adjustment of pH by addition of HCl or NaOH. All measurements were run in triplicate.

Absorption spectra were recorded on a UV Perkin-Elmer Lambda20 spectrophotometer. Luminescence and excitation spectra were obtained either from Perkin-Elmer LS 50 B instrument or a Spex Fluorolog II spectrofluorimeter equipped with a Hamamatsu R928 phototube. The experimental uncertainty in the band

Table 2.
Number average diameter ($\overline{D}_n$), weight average diameter ($\overline{D}_w$), uniformity ratio (U), amount of acid groups per gram microspheres and surface charge density for sample series 1–5

Sample	$\overline{D}_n$ (μm)	$\overline{D}_w$ (μm)	U	COOH/g microsphere (μmol)	Surface charge density (nmol/cm^2)
1a	10.18	11.03	1.08	20.6	3.69
1b	6.15	6.54	1.06	22.0	2.35
1c	2.49	5.38	2.16	29.0	2.01
1d	4.35	4.80	1.10	48.1	37.8
1e	2.60	2.28	1.11	59.2	27.3
2a	2.42	3.89	1.61	27.2	1.42
2b	2.38	2.49	1.05	42.7	1.75
2c	2.36	2.45	1.04	54.2	2.13
2d	1.69	1.73	1.02	62.1	1.78
2e	1.77	1.83	1.04	65.3	1.98
3a	1.64	1.68	1.02	65.4	1.34
3b	2.17	2.22	1.02	58.9	1.94
3c	2.40	2.41	1.01	55.9	2.24
4a	0.80	0.72	1.03	68.7	0.95
4b	0.65	0.67	1.03	60.2	0.65
4c	0.78	0.80	1.02	57.3	0.75
5a	2.13	2.14	1.01	59.2	2.11

maximum for luminescence spectra was 2 nm. Each sample was dissolved in methanol/dichloroethane 1 : 1 (HPLC grade, Aldrich).

Protein adsorption

5.0 mg microspheres was incubated in 1.0 ml sodium phosphate buffer (20 mM, pH 7.4) in the presence of different trypsin concentrations (from 10 to 250 μg/ml) for 2 h at room temperature in low-protein-binding tubes. Then, the microspheres were collected by centrifugation at 15×10^3 rpm for 10 min and the amount of the residual protein in the supernatant was estimated, after filtration on low protein binding filter units (Millex GV100 Millipore), using the bicinchoninic acid (BCA) assay [32]. The amount of adsorbed protein was then calculated as the difference between the added and remaining. Controls were represented by blank samples without protein or microspheres. Experiments were run in triplicate (SD $\leqslant$ 15%).

Cellular uptake of fluorescent microspheres

Human HL3T1 cells (0.5×10^5), a HeLa derivative cell line containing an integrated copy of the HIV-1 long-terminal repeat driving the expression of the chloramphenicol acetyl transferase reporter gene, were seeded in 24-well plates containing a 12-mm glass coverslip and incubated with 30.0 μg/ml of fluorescent 5a microspheres in DMEM (Gibco) with 10% foetal bovine serum added. After

incubation, cells were washed with phosphate-buffered saline (PBS), fixed with 4% cold paraformaldehyde and observed at a confocal laser scanning microscope LSM410 (Zeiss). Image acquisition, recording and filtering were carried out using an Indy 4400 graphic workstation (Silicon Graphics) as previously described [33].

In vivo *inoculation of fluorescent microspheres*

Animal use was according to national guidelines and institutional guidelines. Seven-week-old female BDF mice ($n = 6$) were injected in the quadriceps muscle of the left posterior leg with 1 mg of microspheres **5a** resuspended in 100 μl PBS. As control, mice were also injected in the quadriceps muscle of the right poster leg with 100 μl PBS alone. Fifteen and 30 min after injection, mice were anesthetized intraperitoneally with 100 μl isotonic solution containing 1 mg Inoketan (Virbac) and 200 μg Rompun (Bayer), and killed. Muscle samples at the site of injection were removed, immediately submerged in liquid nitrogen for 1 min and stored at –80°C. Five-μm-thick frozen sections were prepared, fixed with fresh 4% paraformaldehyde for 10 min at room temperature, washed with PBS and coloured with DAPI (0.5 μg/ml; Sigma) for 10 min, which stains the nuclei. After one wash with PBS, the sections were dried with ethanol, mounted in glycerol/PBS containing 1,4-diazabicyclo[2.2.2]octane to retard fading and observed through a fluorescence microscope (Axiophot 100, Zeiss). The green fluorescence (microspheres) was observed with a λ = 450–490 nm, flow through λ = 510 nm and long pass λ = 520 nm filter. The blue fluorescence (DAPI) was observed with a band pass λ = 365 nm, flow through λ = 395 nm and long pass λ = 397 nm filter. For the same microscopic field, green and blue images were taken with a Cool-Snapp CCD camera (RS-Photometrics) and the images were then overlapped using the Adobe Photoshop 5.5 program.

RESULTS AND DISCUSSION

In dispersion polymerization systems, the main parameters affecting the microsphere size are the amount of the steric stabilizer, the medium solvency power and the initiator concentration [21, 34–36]. To get information concerning the effects of these parameters in the present dispersion polymerization system, four sample series were prepared and identified as **1a–e** (5 runs), **2a–e** (5 runs), **3a–c** (3 runs) and **4a–c** (3 runs). In addition, a sample of yellow/green fluorescent microspheres, identified as **5a**, was synthesized using the fluoresceine derivative **1**. All polymerization reactions were performed using MMA as the monomer, AIBN as the free radical initiator and Eudragit L 100-55 as the steric stabilizer either in methanol (series **1**) or in methanol/water mixture (76 : 24 wt% for series **2** and **3**, and 54 : 46 wt% for series **4**). All the reactions were carried out at 60°C for 24 h. In these conditions, yields ranged between 56 and 88%. The reaction yield could be improved by increasing the reaction time but in some cases a decrease of the dispersion stability

was observed, leading to the formation of aggregates. The amount of the steric stabilizer was varied in series **1** and **2**, whereas the effect of the initiator concentration was studied in series **3** and **4**. Moreover, to better understand the effect of the medium solvency on the microsphere characteristics, the composition of the methanol/water dispersion medium was changed. Table 1 reports the reaction conditions for all samples.

As both the monomer and the steric stabilizer are soluble in the reaction medium, the polymerization start in a clear homogeneous system. As soon as the polymerization begins, primary radicals, generated by decomposition of the initiator, grow in the continuous phase with the addition of the monomer units until they reach their critical chain length and precipitate to form nuclei. At this point, which occurs after a nucleation induction time of about 15–30 min, a faint opalescence was observed. These nuclei grow further by capturing monomer, oligo-radicals and stabilizer from the medium [36]. At the end of the reaction, a white and stable dispersion was always obtained. As typical examples, two SEM micrographs of samples **2d** and **5a** are reported in Fig. 1. Table 2 collects some physicochemical characteristics of the samples, including the number average diameter $\overline{D}_n$ and the uniformity ratio U, as well as the amount of carboxylic groups per gram of microspheres and the surface charge density. In general, the size distribution is quite narrow. This indicates a short particle-forming stage and a particle-growth stage free from both formation of new particles and coalescence of existing particles. Figure 2 illustrates the trend of the number average diameter $\overline{D}_n$ for series **1** and **2** as a function of the steric stabilizer amount in the reaction medium. For series **1**, $\overline{D}_n$ is about 10.18 μm, for the sample with the lowest steric stabilizer concentration, but it decreases steeply as the steric stabilizer amount increases. For series **2**, the $\overline{D}_n$ values are definitely lower that those of the corresponding series **1**, and only a slightly pronounced decrease of $\overline{D}_n$ can be observed.

In both series, the amount of carboxylic groups per gram of microspheres increases regularly as the stabilizer amount in solution increases (Fig. 3). The observed size decrease as the stabilizer concentration increases is related to the parallel increase in the grafting rate to the stabilizer which produces more nuclei and, hence, smaller final microspheres and more graft co-polymer, in turn resulting in a greater amount of carboxylic groups at the microsphere surface. Moreover, water is a poorer solvent for PMMA than methanol. Consequently, the critical chain length would decrease and the rate of adsorption of the Eudragit L 100-55-*g*-PMMA co-polymer would increase as the water content in the medium increases, that is, going from series **1** to series **2**. Accordingly, both the rate of nuclei formation and of Eudragit L 100-55-*g*-PMMA adsorption would increase, thus resulting in smaller microspheres and in a greater amount of Eudragit L 100-55 located at the microsphere surface.

Figure 4 reports collectively the trends of $\overline{D}_n$ and the amount of carboxylic groups per gram of microspheres for series **3** and **4**, as a function of the initiator concentration. For series **3**, the microsphere diameter increases with the AIBN

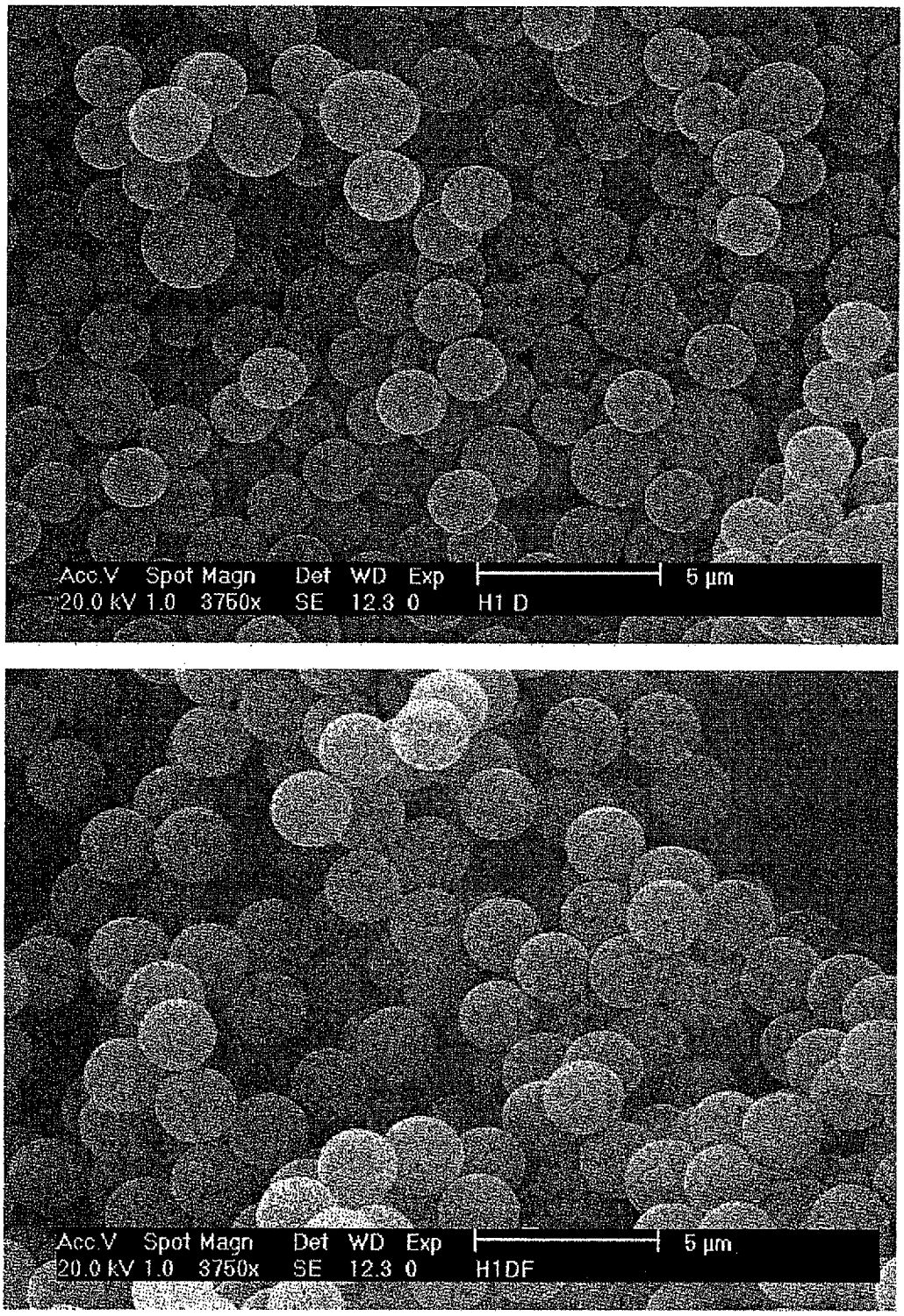

Figure 1. SEM micrographs of samples **2d** (top) and **5a** (bottom).

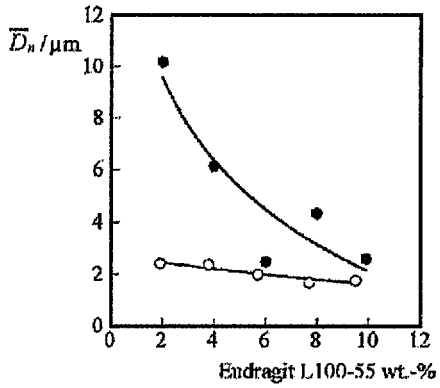

Figure 2. Trend of number average diameter $\overline{D}_n$ for series 1 (●) and 2 (○) as a function of Eudragit L 100-55 content (wt%).

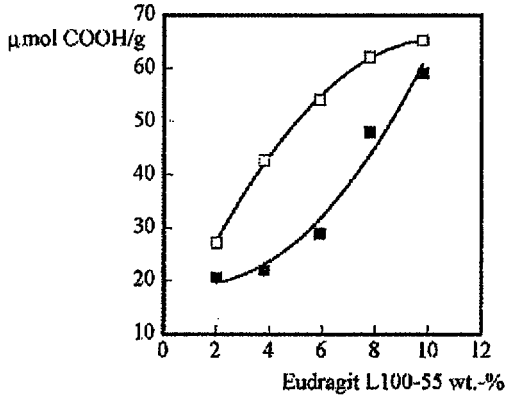

Figure 3. Trend of carboxylic group amount per gram microspheres for series 1 (■) and 2 (□) as a function of Eudragit L 100-55 content (wt%).

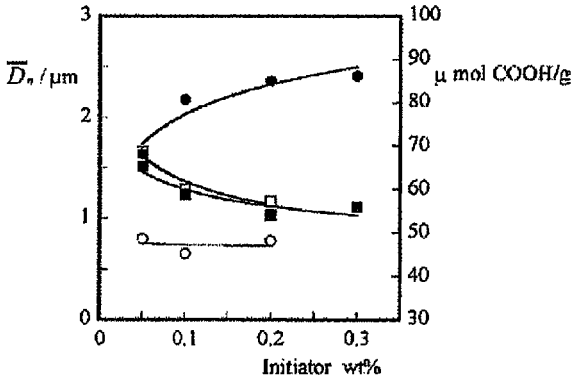

Figure 4. Trend of number average diameter $\overline{D}_n$ (●, series 3; ○, series 4) and of the carboxylic group amount per gram microspheres (■, series 3; □, series 4) as a function of AIBN content (wt%).

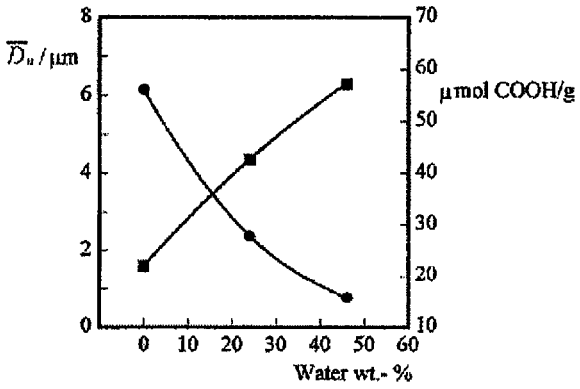

Figure 5. Trend of number average diameter $\overline{D}_n$ (●) and of carboxylic group amount per gram microspheres (■) as a function of the water content (wt%) for samples **1b**, **2b** and **4c**.

content, in agreement to the trend generally reported in the literature [19], whereas for series **4** the size is unaffected by the initiator concentration. For series **3**, the increase in microsphere size can be explained by considering that an increase in the initiator concentration produces shorter PMMA chains which are more soluble so that only a few of them would precipitate to form primary nuclei. The aggregation of many but short macromolecules results, in turn, in few but large final microspheres [26, 37]. This effect is not observed for series **4** probably because the high water content (46 wt%) reduces the critical chain length. The amount of carboxylic groups per gram of microspheres decreases slightly for both series as the AIBN concentration increases.

From the overall picture of the data, it is clear that the main parameter affecting the microsphere size is the solvency power of the reaction medium and, consequently, the amount of water. To highlight the influence of this parameter on the microsphere size, Fig. 5 reports the trends of $\overline{D}_n$ and the amount of carboxylic groups at the microsphere surface for samples **1b**, **2b** and **4c**. These samples were prepared with the same amount of monomer, stabilizer and initiator, the only variation being the water content. As the water content increases, the microsphere size substantially decreases and the amount of carboxylic groups per gram microspheres increases. These effects are in agreement with those previously reported for the MMA dispersion polymerization in a water/methanol mixture using PVP as the steric stabilizer [36]. Water is a poorer solvent for PMMA than methanol and an increase in water content reduces the oligomer solubility, thus decreasing the critical chain length for particles nucleation and increasing the rate of nuclei formation leading to smaller particles.

Figure 6 illustrates the trend of the ionization degree of Eudragit, as estimated from the equilibrium constant (pK_a approx. 5) and the ζ-potential of sample **4b**, chosen as a typical one, as a function of pH. Although it is well known that the ζ-potential decreases as pH increases [38], the similarity of the two sigmoidal curves, presenting very close inflection points, clearly indicates that the Eudragit

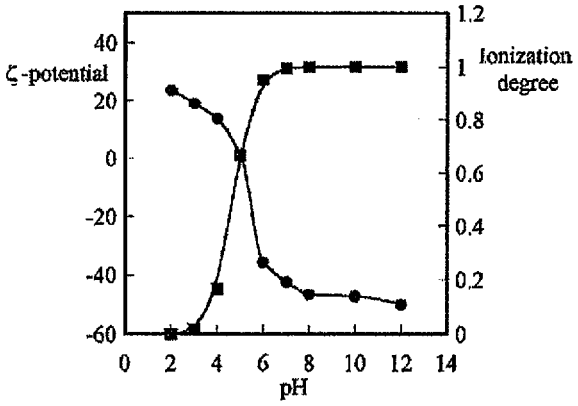

Figure 6. Trend of the ionization degree and ζ-potential as a function of pH for sample **4b**.

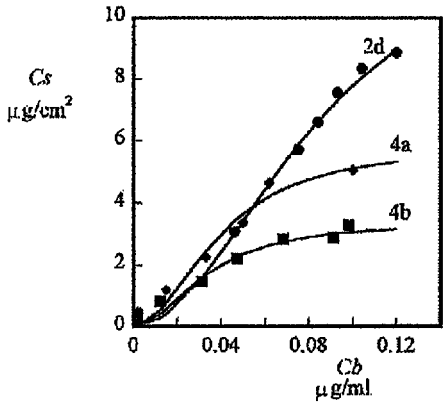

Figure 7. Adsorption isotherms of trypsin on microsphere samples **4a**, **4b** and **2d** in 20 mM sodium phosphate buffer (pH 7.4).

is located at the microsphere surface, thus further confirming that the dispersion polymerization process is highly effective in providing microspheres endowed with a specific and tuneable surface.

The protein adsorption behaviour on the above microspheres was investigated using trypsin, a well known, cheap and easily available model protein whose molecular weight and p*I* (9.74) are similar to Tat (p*I* 10.22). Microspheres **2d**, **4a** and **4b** were chosen because of their different size and surface charge density.

Figure 7 illustrates the trend of trypsin adsorption at physiological pH as a function of the equilibrium trypsin amount. The adsorption isotherms were fitted according to the Langmuir–Freundlich equation (1) [35, 36]:

$$C_s = \frac{C_m K C_b^{1/n}}{1 + K C_b^{1/n}}, \tag{1}$$

by means of a non-linear regression method.

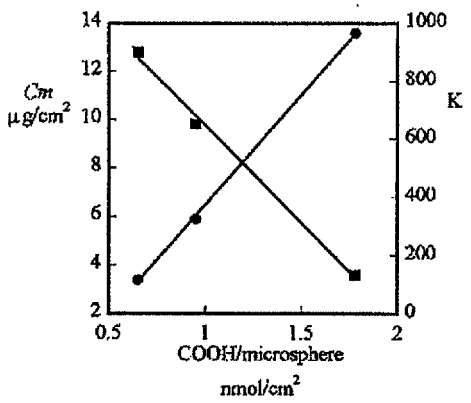

Figure 8. Trend of the Langmuir–Freundlich parameters C_m and K as a function of the carboxylic groups surface density on microsphere samples **4a**, **4b** and **2d**.

In this equation, C_s is the amount of adsorbed trypsin per unit surface area, C_b is the bulk concentration of trypsin, C_m is the plateau adsorbed amount (maximum amount of adsorption) and K is the equilibium adsorption constant. In the Langmuir isotherm, K is the ratio of the adsorption to the desorption rate constant. In the Langmuir–Freundlich, this definition does not apply but its meaning is almost the same. A large value of K represents a faster rate of adsorption over desorption rate. The value of n was fixed at 0.5 in the various calculations [39]. Figure 8 illustrates the trend of C_m and K as functions of the carboxylic group surface density. The increase of C_m indicates that the adsorption is mainly governed by polar interactions, namely hydrogen bonds or electrostatic interactions. In contrast, K decreases as the carboxylic group surface density increases. A similar effect was also observed for microspheres bearing sulphonic or carboxylic groups at the surface and it was explained considering a critical balance between hydrophobic and hydrophilic forces [40]. In particular, it was suggested that the adsorption rate due to hydrophobic forces is faster than the one due to hydrophilic forces. Consequently, the overall adsorption to desorption rate should decrease as the hydrophilic contribution increases. Although this explanation seems quite reasonable, other experiments should be performed to clarify this critical point.

To assess the capability of these microspheres to be taken up by the cells, a fluorescent core–shell microsphere sample (**5a**) was also prepared. Since fluorescent particles obtained by simple dye adsorption at the surface can give rise to desorption of dye and loss of fluorescent emission following exposure to light and during *in vitro* experiments, a sample was prepared using the reactive fluoresceine derivative **1**. Although allylic monomers do not undergo radical polymerization, they are able to co-polymerize or at least to be included in the polymer chain as a terminal group. Accordingly, the microsphere sample **5a** was prepared by running the dispersion polymerization under the same experimental conditions as sample **2c** with the addition of a small amount of the fluorescent monomer **1**. A microsphere sample

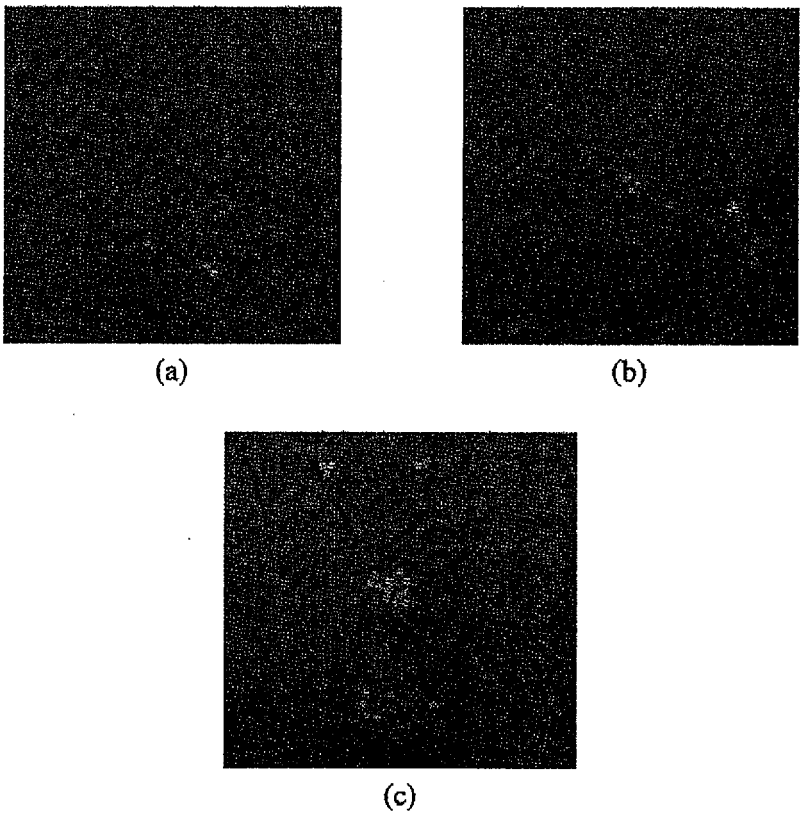

Figure 9. Confocal microscopy images of fluorescent microspheres **5a** uptake by HL3T1 cells, after 30 min (a), 6 h (b) and 24 h (c) incubation.

with $\overline{D}_n = 2.13$ μm and $U = 1.01$ was obtained. After the conventional purification procedure, a small amount of sample **5a** was dissolved in chloroform and precipitated in methanol. The polymeric material appeared fluorescent, whereas no trace of fluorescence was observed in the precipitation medium. This demonstrates that the fluorescent units deriving from **1** were covalently bound to the PMMA constituting the inner core of the microspheres. Sample **5a** presents an emission maximum at 535 nm ($\lambda_{exc} = 488$ nm) and good photostability. The photoemission intensity of **5a** in organic solution remained high and unaffected after exposure to daylight for 30 days, whereas that of fluorescein was reduced by approximately 50% under the same conditions. This is in agreement with the fluorescence emission behaviour of commercially available particles with dyes covalently bound inside the polymeric matrix [41]. Cellular uptake experiments in human epithelial cells (HL3T1) were then carried out in the presence of sample **5a.** The analysis of the cells, under a confocal microscope, indicated that the fluorescent microspheres were rapidly and completely taken up in 24 h (Fig. 9). Of note, no significant difference in cellular uptake is detected when Tat protein is adsorbed onto the microsphere surface [30].

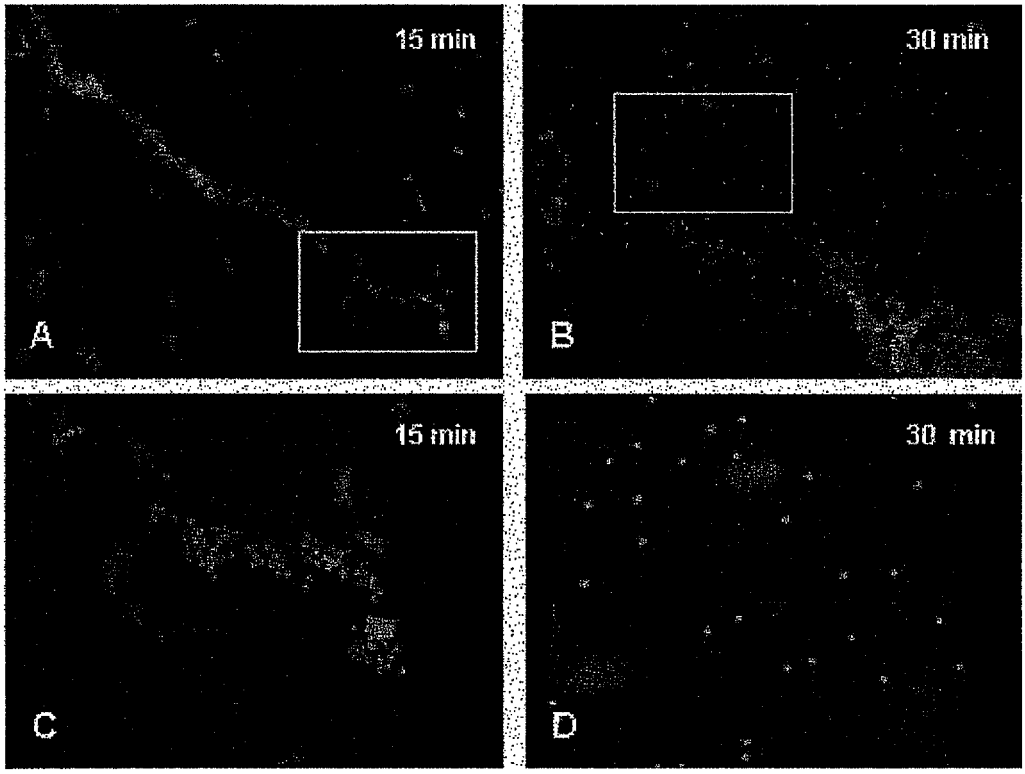

Figure 10. Analysis at the site of injection of cellular uptake of **5a** fluorescent microspheres, 15 min (panels A and C) and 30 min (panels B and D) after inoculation. For the same microscopic field, green (**5a** fluorescent microspheres) and blue (DAPI) overlapped images are shown. (A, B) 40× magnification, (C, D) 100× magnification of the images shown in the white square of panels A and B, respectively.

Finally, to determine whether the microspheres are taken up by the cells *in vivo*, mice were injected intramuscularly with sample **5a** and killed 15 or 30 min after injection. The muscle at the site of injection was then observed under a fluorescent microscope. As shown in Fig. 10, microspheres were taken up by muscle cells after injection, confirming the *in vitro* internalization results described above. These data clearly indicate that the fluorescent sample **5a** represents a useful tool for *in vivo* studies.

CONCLUSIONS

In the present report, core–shell microspheres in the micron scale range were prepared by dispersion polymerization. An appropriate selection of the experimental parameters and in particular of the initiator and stabilizer amount and the medium solvency power allows a monodisperse sample as large as 600 nm to be prepared in which the shell is constituted by the steric stabilizer bearing carboxylic groups able to interact with proteins mainly *via* ionic interactions. To this purpose, low initia-

tor concentration, high steric stabilizer amount and a low solvency power medium must be employed. The described microspheres are able to efficiently bind trypsin, chosen as model protein for Tat. The amount of adsorbed protein is linearly related to the carboxylic group amount. Eventually, these microspheres were shown to be readily taken up by non-phagocytic cells, *in vitro* and *in vivo*. As a final remark, the dispersion polymerization reaction is versatile in providing core–shell microspheres with highly differentiated characteristics [42]. The synthetic procedure allows large-scale preparation of reproducible, stable and homogeneous microsphere stocks, and a wide modulation of the outer shell, so that specific and reversible adsorption of antigens with varying hydrophobicity, molar mass and isoelectric point can be envisaged. Formation of the protein–microsphere complexes is easy and fast, since they spontaneously assemble in aqueous solution after incubation of the two components at room temperature and no purification steps are required. These features, together with the observation that the antigen–microsphere complexes preserve the protein native conformation and biological activity, thus increasing the protein stability [30], and induce potent antigen-specific immune responses in mice (data not shown), indicate that these novel microspheres represent suitable adjuvants for the development of novel and safe protein-based vaccines with increased shelf-life and capable of triggering long-term specific immunity.

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